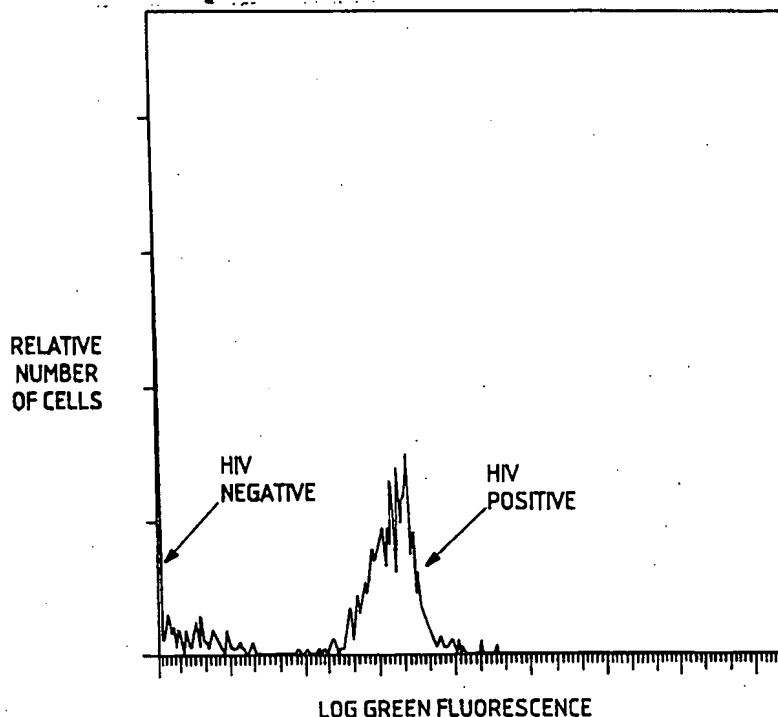


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(54) Title: METHOD OF DETECTING AMPLIFIED NUCLEIC ACID SEQUENCES IN CELLS BY FLOW CYTOMETRY



## (57) Abstract

The field of this invention is the detection and isolation of specific amplified DNA sequences by flow cytometry. More particularly, this invention relates to the detection of these specific amplified DNA sequences in cells so as to allow quantitation of viral burden of patients infected with a virus. The method is particularly adapted to detection of HIV-1 proviral DNA sequences and the assessment of activity of the virus in a cell. The figure illustrates the detection of HIV-1 negative and HIV-1 positive populations of cells by this flow cytometric method.

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# METHOD OF DETECTING AMPLIFIED NUCLEIC ACID SEQUENCES IN CELLS BY FLOW CYTOMETRY

## Field of the Invention

The field of this invention is the detection of amplified nucleic acid sequences by flow cytometry. More particularly, this invention relates to the detection of these amplified nucleic acid sequences in cells, so as to allow quantitation of viral burden of patients infected with a virus. Additionally this invention will allow identification of aberrant cellular genes related to a genotypic carrier state or related to malignancy and of fetal cells in the maternal circulation that arise during maternal/fetal exchange.

## Background of the Invention

Detection of viral nucleic acids in specimens comprising body fluids or tissues can be difficult because of the small quantity of viral DNA present in the specimen and/or because of the presence of other interfering materials, including DNA from a different source. These limitations may be overcome by employing the analytic method referred to as the polymerase chain reaction (PCR) technique. By this technique, selective enrichment of a specific DNA sequence can be achieved by exponential amplification of the target sequence. [See Mullis, et al., Methods Enzymo., 155, 335 (1987); and Saiki, et al., Science, 230, 1350 (1985)].

To facilitate PCR amplification, pairs of oligonucleotide primers may be employed as described in United States Patents 4,683,202 and 4,683,195. The primers are designed to hybridize with sequences that flank the target DNA. Following in vitro amplification, the amplified target sequence is detected by a hybridizing, target-specific probe. For

example, this analytical procedure has been used for direct detection of HIV-1 (AIDS virus), as described by Ou, et al., Science, 238, 295-297 (January 15, 1988). The amplification cycles are facilitated by using a polymerase which is thermally stable in incubations up to 95°C, as described by Saiki, et al., Science, 239, 487-491 (January 29, 1988).

Generally, specific amplified nucleic acids are detected by hybridization with labeled nucleic acid sequences complementary to a region within the amplified nucleic acid. Thus, the whole population of amplified nucleic acids are simultaneously detected.

Other measurement techniques, however, allow the analysis of individual cells. These techniques included microscopy and flow cytometry. Flow cytometry, involves analyzing cells or cellular fractions suspended in a solution that are stained with fluorescent dyes. In flow cytometry, cells are forced in a narrow stream through a path of laser light. The cells pass the laser beam in single file at a rate up to several thousand per second. When cells enter the light, they scatter light or emit fluorescence. As each cell passes through the light source, its optical properties are quantified and stored with this technique. A large number of cells can be measured characterized individually in a short period of time.

Van den Engh et al., U.S. Patent No. 4,770,992 show detection of DNA sequences in chromatin by flow cytometry. The method disclosed by Van den Engh et al., however, does not allow for the detection of short target sequences or nucleic acids located in the cytoplasm. To make these assessments an intact cell must be analyzed.

Quantification of viral burden in patients with an infection is relevant for prognostic and therapeutic

purposes. For example, several studies have attempted to quantify Human Immunodeficiency Virus (HIV-1) DNA or RNA using coamplification of HIV-1 GAG and HLA-DQ- $\alpha$  [See T. Lee, F.J. Sunzeri, L.H. Tobler, et al., Aids 5, 683 (1991)], quantitative RNA PCR [See L.Q. Zhang, P. Simmonds, C.A. Ludlam, A.J.L. Brown, Aids 5, 675 (1991)], and quantitative DNA PCR following cell sorting [See S.M. Schnittman, M.C. Psallidopoulos, H.C. Lane, et al., Science 245, 305 (1989)]. In addition, histologic methods such as in situ hybridization using cRNA probes complementary to HIV-1 RNA [See M.E. Harper, L.M. Marselle, R.C. Gallo, F. Wong-Staal, Proc Natl Acad Sci USA 83, 772 (1986)] and in situ PCR for HIV-1 proviral DNA [See O. Basgasra, S.P. Hauptman, H.W. Lischner, M. Sachs, R.J. Pomerantz, N Engl J Med 326, 1385 (1992)] have been used to directly identify infected peripheral mononuclear cells isolated from patients. Due to the markedly discordant results in these studies only limited insight is gained into the percent of infected cells in HIV-1 patients with estimates ranging anywhere from 10% of cells in symptomatic patients containing HIV-1 DNA [See K. Hsia, S.A. Spector, J Infect Dis 164, 470 (1991)] to between 1 in 100 and 1 in 100,000 cells in asymptomatic carriers containing HIV-1 (See S.M. Schnittman, J.J. Greenhouse, M.C. Psallidopoulos, Ann Intern Med 113, 438 (1990)]. Additionally, the lack of consistent data confuses interpretations of the role that this determination plays in disease progression and prognosis.

Previous attempts to combine nucleic acid hybridization with flow cytometry have been restricted by target copy number or sequence specificity. Solution hybridization and flow cytometric detection of positively hybridized nuclei has been reported

utilizing either total genomic DNA [See G. Dudin, T. Cremer, M. Schardin et al., Hum Genet 76, 290 (1987)] or highly repetitive chromosome specific sequences [See D. Pinkel, J.W. Gray, B. Trask, Cold Spring Harbor Symposia on Quantitative Biology, Vol. LI., 151 (1986)] as probes. Additionally, flow cytometric detection of hybridization to ribosomal RNA has been successful [See J.G. Bauman et al., Cytometry 9:515-524 (1988)] and detection of a high abundance mRNA;  $\alpha$ -actin in L929 cells has been reported [See E.A. Timm, Jr., C.C. Stewart, Biofeedback 12, 363 (1992)].

In this application, we describe a technique which combines the sensitivity of in situ polymerase chain reaction and the specificity of nucleic acid hybridization with the rapid and quantitative single cell analytic capability of flow cytometry. Unlike in situ polymerase chain reaction technique performed on cells adhered to slides, the solution based technique described in this application potentially allows for the multiparameter analysis of large numbers of cells by flow cytometry and further characterization following cell sorting. Using this technique, we have detected a single HIV-1 proviral sequence per cell in an HIV-1 positive cell line and HIV-1 proviral sequences in HIV-1 infected patients.

#### Summary of the Invention

It is an object of this invention to provide a diagnostic method to assess the viral burden of patients infected with a virus such as HIV-1, aberrant genes/translocations related to malignancy and birth defects and of maternal/fetal exchange. These objects are accomplished by the methods described in this application.

The present invention provides a method of detecting amplified nucleic acid sequences in cells

using flow cytometry. More specifically, this invention provides a method to detect specific preselected nucleic acid sequences in cells by fluorescence activated cytometry involving the steps of a) isolating a class of cells; b) incubating the cells in a water soluble fixative to stabilize cells in suspension; c) amplifying specific nucleic acid sequences within cells in the presence of deoxyribonucleoside triphosphates coupled to molecules that prevent diffusion of amplified product from the cells; d) contacting the solution of cells containing amplified nucleic acids with labeled nucleic acid probes complementary to the specific amplified nucleic acid sequence and e) detecting the labeled nucleic acid probes by fluorescence activated flow cytometry so to identify cells containing said preselected nucleic acid sequences. Both DNA and RNA nucleic acid sequences can be detected.

Still more specifically this invention provides a method to detect HIV-1 proviral DNA, RNA and mRNA in peripheral mononuclear cells by fluorescence activated flow cytometry by a) isolating peripheral blood mononuclear cells; b) incubating the peripheral blood mononuclear cells with a water soluble fixative to form a suspension; c) amplifying specific DNA sequences in the suspension of peripheral blood mononuclear cells in the presence of deoxyribonucleoside triphosphates coupled to molecules that prevent diffusion of amplified product from the cells; d) contacting the solution of peripheral mononuclear cells containing amplified proviral HIV-1 DNA with labeled nucleic acid probes complementary to the proviral HIV-1 DNA sequence; and e) detecting the labeled nucleic acid probes by fluorescence activated flow cytometry so to identify cells containing said HIV-1 proviral DNA.

Similarly, this method can be practiced to detect HIV-1 mRNA sequences.

In these methods, the nucleic acid is retained in the cells using the water soluble fixative STF (Streck Laboratories) and also by coupling certain deoxyribonucleoside triphosphates to large molecules such as steroids. More specifically, this invention provides the steroid digoxigenin coupled to the deoxyribonucleoside triphosphate UTP.

#### Brief Describing Figures

Figure 1. Shows in situ PCR using primers SK38/39 on 8E5/LAV cells diluted to 50% using seronegative peripheral mononuclear cells. Amplificate remains in positive cells (dark brown) and negative cells contain no amplified product (light green). Color development was achieved using an alkaline phosphatase conjugated anti-digoxigenin antibody which binds to digoxigenin incorporated in the amplificate. The substrate NBT/xphosphate precipitates to form a brown product. Cells were counterstained with Fast Green.

Figure 2. Standard curve of 8E5/LAV cells (HIV-1 positive) diluted with seronegative peripheral mononucleic cells as determined by in situ PCR and flow cytometric analysis (y-axis).

Figure 3. Fluorescence histogram showing HIV-1 positive and HIV-1 negative cells quantitated by in situ PCR and flow cytometric analysis.

#### Detailed Description of the Invention and Best Mode

In accordance with the present invention amplified nucleic acid sequences in cells are detected using flow cytometry. When working with a flow cytometer, generally a specific class of cells are transported through the instrument. This invention specifically relates to the detection of certain preselected nucleic acid sequences in peripheral blood mononuclear cells,



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but other classes of cells such as monocytes, thymocytes and digested tissues can also be used.

The isolated cells are adjusted to a desired concentration for amplification such as  $1 \times 10^6$  cells/ml. The cells are treated with a water soluble fixative STF (Streck Laboratories). STF is a proprietary fixative agent containing acetic acid and zinc. The dilution that we found to be useful for peripheral blood mononuclear cells was about the same as recommended for general tissue staining, i.e., about 1.5x, although, the correct concentration must be determined empirically for each type of cell examined. In order to assess concentration of the fixative agent both the morphology of the cell and the efficacy of amplification are reviewed.

In the context of this invention we will discuss amplification in terms of the polymerase chain reaction (PCR) technique (U.S. Patent Nos. 4,683,202 and 4,683,195 hereby incorporated by reference), but it should be noted that other amplification techniques may also be used to practice this invention. See e.g. Erlich et al. EP 0 237 362, Dettagupta et al. EP 0 297 379, Becker EP 0 300 769, Burg et al. 0 310 229, Collins EP 0 328 822, Davey 0 329 822, Loewy et al. EP 0 369 775, EP 0 371 437, Gingeras EP 0 373 960, and Rose et al. EP 0 379 639.

The polymerase chain reaction amplification procedure was conducted as disclosed in U.S. Patent No. 4,683,202 and 4,683,195. To facilitate retaining amplified product within the cell after application of proteinase K and thermal cycling, deoxyribonucleoside triphosphates are coupled to bulky molecules such as biotin or digoxigenin. We have found that the incorporation of these molecules during amplification

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provides a way to detect amplified DNA in a cell in solution because these molecules function to prevent amplified product from leaking out of the cell.

Amplification localization and optimization of cycling parameters were evaluated using an antibody to the compound coupled to the deoxyribonucleoside triphosphates. In particular, for detection of HIV-1-DNA certain primers SK38/39 (GAG) and SK68/69 (ENV) provides the greatest sensitivity and specificity. Additionally, the concentration of magnesium also affected sensitivity and specificity. (Table 1)

This technique can be used to detect specific nucleic acid sequences such as HIV-1 proviral DNA, HIV-1 RNA, HLA-DQ $\alpha$ , and potentially T-cell receptor and B-cell gene rearrangements.

#### EXAMPLE 1

##### HIV-1 Proviral DNA

Cell Lines and Viruses: 8E5/LAV cells are an established cell line containing a single copy of HIV-1 proviral DNA. 8E5/LAV is available from ERC Bioservices Corporation -- catalog number 95. This reagent was obtained through the AIDS Research End Reference Reagent Program, Division of AIDS, NIAID, NIHS 8E5/LAV from Dr. Thomas Folks. Folks, T.M., Powell, D., Lightfoote, M. Koenig, S., Fauci, A.S., Benn, S., Rabson A., Daugherty, D., Gendelman, H.E., Hoggan, M.D., Venkatesan, S., and Martin, M.A., Biological and biochemical characterization of a cloned Leu-3-cell surviving infection with the acquired immune deficiency syndrome retrovirus. J. Exp. Med. 164: 280-290, 1986. The growth characteristics are provided in a data sheet that accompanies a shipment of the cell line.

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8E5/LAV, an established cell line having one HIV-1 proviral DNA molecule per cell, was used to prepare a copy number standard curve for polymerase chain reaction amplification. Low passaged 8E5/LAV cells are maintained in suspension culture in RPMI 1640 media (Gibco Laboratories) supplemented with 20% fetal bovine serum (Hyclone Labs), 2mM L-glutamine, and penicillin (100 units/ml) streptomycin (100 micrograms) in a humidified incubation with a 5% CO<sub>2</sub> atmosphere.

HIV-1 negative peripheral mononuclear cells were separated from the whole blood of normal healthy donors who were in a low risk for AIDS and who had been screened as negative for HIV-1 antigen/antibody and for Hepatitis B surface antigen. Mononuclear cells were separated from heparinsized whole blood by centrifugation on a LYMPHOCYTE SEPARATOR medium (Organon Teknike Corporation) gradient. The layer containing the peripheral mononuclear cells was removed and washed three times with Dulbecco's phosphate buffered saline, magnesium and calcium free (D-PBS, Gibco Laboratories). Cell suspensions of the positive control cells, 8E5/LAV, negative cells, and HIV-1 positive clinical samples was quantified by hemocytometer counting until duplicate counts were within 5% of each other. The concentration of cell suspensions was adjusted to  $1 \times 10^6$  cells per milliliter with D-PBS. Four hundred microliters of this suspension is equivalent to 400,000 cells and in the case of 8E5/LAV cells is equivalent to 400,000 copy numbers of HIV-1 proviral DNA. Standard of curve dilution medium consisted of the HIV-1 negative peripheral blood mononuclear cells ( $1 \times 10^6$  cells/milliliter) D-PBS. Several dilutions of 8E5/LAV cells were made in the standard curve dilution medium of negative peripheral mononuclear cells to obtain

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standard curve point of 50,000, 100,000, 200,000, and 400,000 copy numbers. The zero copy number point was HIV-1 negative peripheral mononuclear cells alone. Using a dilution medium of negative peripheral blood mononuclear cells ensured a consistent total number of cells in each polymerase chain reaction amplification tube. Four hundred microliters of each standard curve dilution and clinical sample was aliquoted in 0.5ml microfuge tube (Eppendorf) for in situ polymerase chain reaction.

Peripheral blood mononuclear cells were isolated from fresh heparinized blood layered on a HISTOPAQUE 077 (Sigma, St. Louis, MO) density gradient. This gradient was centrifuged for 30 min. in GH-37 rotor at 1600 rpm at room temperature. The turbid mononuclear layer was removed and transferred to clean 15 ml. conical tube. The cells were washed twice with three volumes of RPMI and once with phosphate buffered saline (pH 7.6).

In situ Polymerase Chain Reaction: cell samples were adjusted to a concentration of  $1 \times 10^6$  cells/ml and 400  $\mu$ l of each sample was pelleted at 1500 rpm for two min. After removal of the supernatant, the cells were resuspended in 50  $\mu$ l of STF (Streck Laboratories, Omaha, NE) fixative and incubated at room temperature for fifteen min. Cells were again pelleted at 1500 rpm for two min. resuspended in 25 $\mu$ l of 1 $\mu$ g/ml proteinase K in 0.1M Tris HCL, 50mM EDTA (pH 8.0), and incubated at 37°C. for fifteen min. Cells were pelleted as above, washed with phosphate buffered saline (pH 7.4) and placed on ice. 190 $\mu$ l of polymerase chain reaction mixture (10mM Tris HCL pH 8.3, 50mM KCL, 1.5mM MgCl, 0.25mM dATP, dCTP, dGTP, 0.14mM dTTP, 4.3 $\mu$ M dUTP-11-digoxigenin (Genius 1 DNA labeling and Detection Kit (hereby incorporated by

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reference), 100 pmole each forward and reverse primer (SK 38/39 Primers) 1.0 $\mu$ l (5 $\mu$ ) Taq polymerase (Amplitaq, Perkin Elmer, Norwalk, CT) and gelatin 0.001% w/v was added to said sample. (Samples were placed in a Perkin Elmer Cetus automated thermocycler once block temperature reached 80°C, then cycled. Thermal cycling parameters (optimal) were as follows: denaturation-94°C, one min., reannealing 58°C, two min., extension-74°C. 1.5 min. with five sec. added for each successive extension cycle. Cells were cycled for 40 cycles and stored at 4°C after cycling if necessary. Amplification localization and optimization of cycling parameters were evaluated using an antidigoxigenin alkaline phosphatase conjugated antibody. See Figure 1 and Table 1. Briefly, amplified cells were cytospun onto poly-L-lysine coated slides, washed with phosphate buffered saline pH 7.4 and incubated with the conjugated antibody for two hours at 37°C. Cells were washed as above and incubated with substrate (NBT/X-phosphate) for 10 min. at room temperature. Cells were counterstained with FAST GREEN (Rowley Biochemical Institute, Rowley, MA) and coverslipped.

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TABLE 1

OPTIMIZATION OF IN SITU PCR CYCLING CONDITIONS  
USING IMMUNOHISTOCHEMICAL DEVELOPMENT

PRIMERS	[Mg]	Sensitivity	Specificity
GAG	1.5mM	100%	98%
(SK38/39)			
	2.25mM	100%	95%
ENV	1.5mM	98%	94%
(SK68/69)			
	2.25mM	100%	91%

Solution hybridization. Sequence specific oligonucleotide probes (Applied Biosystems, San Diego, CA) SK19-FITC) containing multiple fluorescence tagged nucleotides were added to the polymerase chain reaction tubes (400 pmol/tube) along with 10  $\mu$ g/ml sonicated herring sperm DNA. Tubes were heated to 94°C for two min. and hybridization was performed for two hrs. at 56°C. Cells were washed under high stringency for thirty min. with 2x SSC/50% formamide/500 $\mu$ g/ml bovine serum albumin at 42°C, 30 minutes with 1x SSC/50% formamide/500 $\mu$ g/ml bovine serum albumin at 42°C, thirty min. with 1x SSC/500 $\mu$ g/ml bovine serum albumin at room temperature, and briefly with phosphate buffered saline temperature. Cells were resuspended in phosphate buffered saline pH 8.3 and counterstained for flow cytometric analysis with 1 $\mu$ g/ml propidium iodide.

Flow cytometric analysis. All samples were filtered through a 7  $\mu$ m nylon mesh just prior to analysis. Samples were analyzed on the Coulter Electronica PROFILE II flow cytometer with POWERPAK option (Coulter Electronica, Inc., Hialeah, FL.) at

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Veterans Lakesize Medical Center. Laser excitation was 15mW at 488 nm and the standard optical filter configuration was utilized for fluorescence light detection (488 nm dichroic, 457-502 nm long pass laser blocking, 550 nm dichroic, 525 band-pass FITC fluorescence), 600 nm dichroic, and 635 nm band-pass (propidium iodide fluorescence filter). Instrument sensitivity was standardized before each experiment employing Immuno-Bright calibration beads (Coulter Source, Marriette, GA). Color compensation circuitry was adjusted using FITC only, PI only, and dual stained 100% HIV-1-positive cell samples. Standard curve showing the linearity of this assay is shown in Figure 2. Sensitivity and specificity of this technique using the solution hybridization was 99.6% and 99.2% respectively (Figure 3).

#### Three-Color Analysis of Cell Surface Markers.

Monoclonal antibodies OKT4-FITC (Ortho diagnostics), CD3-PerCP (Coulter Diagnostics), and CD2-PE (Coulter Diagnostics) were employed for cell surface phenotyping. Peripheral blood mononuclear cells were resuspended in 100  $\mu$ l phosphate buffered saline (pH 7.4) and all antibodies were added at concentration recommended by the manufacturer. After thirty minutes incubation at room temperature the cells were fixed in 1% paraformaldehyde and analyzed by flow cytometry. The results of this analysis is shown in Table 2.

TABLE 2

In Situ PCR for HIV-1 Proviral DNA  
Correlation with CD4 Counts

<u>Patient Number</u>	<u>CD4 (% of PBMC)</u>	<u>HIV-1 DNA+ (% of PBMC)</u>
1	14.3	15.0
2	28.3	3.2
3	35.2	1.4
4	0.1	4.2
5	0.2	0.0

The data presented in this table shows the detection of HIV-1 proviral sequences in HIV-1 infected patients. Patient No. 1 has symptoms of the disease and shows about 14% of the mononuclear cells being CD4+T-cells. Similarly, 15% of the mononuclear cells contain a HIV-1 proviral sequence. Patient No. 2 and 3 are asymptomatic and have higher T-cells percentages and lower HIV-1 proviral DNA percentages. Patients 4 and 5 are near death and show low T-cells and HIV-1 proviral DNA percentage. All patients are HIV-1 antibody positive.

EXAMPLE 2

In Situ RNA PCR

Lymphocytes were aliquoted and treated with fixative and proteinase K as described for DNA PCR, although all solutions were prepared with 0.1% Diethyl pyrocarbonate (DEPC) treated analytical reagent water (Mallinckrodt). Glassware and plasticware were used also treated with 0.1% DEPC prior to autoclaving.



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To each 400,000 cell sample, 40 $\mu$ l of reaction mixture for reverse transcription was added (10.0 units thermostable rTth DNA Polymerase (Perkin Elmer Cetus) 90mM KCl, 100mM Tris-HCl pH 8.3, 1.0mM MnCl<sub>2</sub>, 200 $\mu$ M each dGTP, dATP, dCTP, 125 $\mu$ M dTTP, 4 $\mu$ M dUTP-11-digoxigenin (Boehringer Mannheim), RNase Inhibitor 40 units (Perkin Elmer Cetus), 100 pmoles downstream primer. Samples were incubated for 15 minutes at 70°C and placed on ice.

160 $\mu$ l of PCR reaction mixture was then added (100mMKCl, 10mM Tris-HCl pH 8.3, 0.75 mM EGTA, 0.05% Tween 20, 5.0% (v/v) glycerol (Chelating buffer-Perkin Elmer Cetus) 2mM MgCl<sub>2</sub>, 100 pmoles upstream primer. Samples were taken from ice and placed in an automated thermal cycler with block temperature at 80°C. Cycling was then performed as previously described.

The primers used for RNA amplification (MF111, MF126) were provided by Dr. M. Furtado.

5869-5886 MF111 GCGAATTCATGGAKCCAGTAGATCCTAGACTA  
(Sequence Id. No. 1 and 2) 8760-8733 MF126  
GCTCTAGACTATCTGTCCCCTCAGCTACTGCTATGG

(Sequence Id. No. 3) flank a major splice site within the mRNA species which encodes the TAT protein.

Solution hybridization was then performed as described for DNA with a fluorescently labeled oligonucleotide probe which crosses the mRNA splice site (MFA-1) MFA-1 TTCTCTATCAAAGCAACCCACCTCCCAATC  
(Sequence Id. No. 4).

Cells used as positive controls were CEM cells infected with NL4-3 HIV-1.

Although the invention has been described primarily in connection with special and preferred embodiments, it will be understood that it is capable of modification without departing from the scope of the invention. The following claims are intended to cover

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all variations, uses, or adaptations of the invention, following, in general, the principles thereof and including such departures from the present disclosure as come within known or customary practice in the field to which the invention pertains, or as are obvious to persons skilled in the field.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: WOLINSKY, STEVEN  
PATTERSON, BRUCE  
TILL, MICHELLE

(ii) TITLE OF INVENTION: METHOD OF DETECTING AMPLIFIED  
NUCLEIC ACID SEQUENCES IN CELLS BY FLOW  
CYTOMETRY

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:

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(D) STATE: ILLINOIS  
(E) COUNTRY: USA  
(F) ZIP: 60606-4002

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
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(vi) CURRENT APPLICATION DATA:

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(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: FENTRESS, SUSAN B  
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(C) REFERENCE/DOCKET NUMBER: NU-9218

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 312/456-8000

-18-

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCGAATTCAT GGAGCCAGTA GATCCTAGAC TA

32

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCGAATTCAT GGATCCAGTA GATCCTAGAC TA

32

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCTCTAGACT ATCTGTCCCC TCAGCTACTG CTATGG

36

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: unknown

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TTCTCTATCA AAGCAACCCA CCTCCCAATC

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## WE CLAIM:

1. A method to detect specific preselected nucleic acid sequences in a cell by fluorescence activated cytometry comprising the steps of:
  - a) isolating a class of cells;
  - b) incubating said cells with a water soluble fixative to form a suspension;
  - c) amplifying specific nucleic acid sequences in said suspension of cells in the presence of deoxyribonucleoside triphosphates coupled to molecules that prevent diffusion of amplified products from said cells;
  - d) contacting said solution of cells containing amplified nucleic acid with labeled nucleic acid probes complementary to said specific nucleic acid sequences.
  - e) detecting said labeled nucleic acid probes by fluorescence activated flow cytometry so to identify cells containing said preselected nucleic acid sequences.
2. The method of Claim 1 wherein said class of cells is peripheral mononuclear cells.
3. The method of Claim 1 wherein said water soluble fixative is STF (Streck Laboratories).
4. The method of Claim 1 wherein said deoxyribonucleoside triphosphate is coupled to digoxigenin (Boehringer Mannheim).
5. The method of Claim 4 wherein said triphosphate compound is dUTP.
6. The method of Claim 1 wherein said specific nucleic acid is HIV-1 proviral DNA.
7. The method of Claim 1 wherein said nucleic acid is DNA.

8. The method of Claim 1 wherein said nucleic acid is RNA.

9. The method of Claim 1 wherein said specific nucleic acid is HIV-1 RNA.

10. A method to detect HIV-1 proviral DNA sequences in peripheral mononuclear cells by fluorescence activated flow cytometry comprising the steps of:

- a) isolating said peripheral mononuclear cells;
- b) incubating said peripheral mononuclear cells with a water soluble fixative to form a solution;
- c) amplifying said HIV-1 proviral DNA sequence in said solution of cells in the presence of deoxyribonucleoside triphosphates coupled to molecules that prevent the leakage of cellular materials from said cells;
- d) contacting said solution of cells containing amplified HIV-1 proviral DNA sequence with labeled nucleic acid probes complementary to said nucleic acid sequences; and
- e) detecting said labeled HIV-1 proviral nucleic acid probes by fluorescence activated flow cytometry so to identify cells containing said HIV-1 proviral DNA sequences.

11. The method of Claim 10 wherein said fixative agent is STF (Streck Laboratories).

12. The method of Claim 10 wherein said deoxyribonucleoside triphosphates are coupled to digoxigenin.

13. The method of Claim 12 wherein said triphosphate compound is dUTP.

14. A method to detect HIV-1 proviral RNA sequences in peripheral mononuclear cells by fluorescence activated flow cytometry comprising the

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steps of:

- a) isolating said periperal mononuclear cells;
- b) incubating said periperal mononuclear cells with a water soluble fixative to form a solution;
- c) amplifying said HIV-1 RNA sequence in said solution of cells in the presence of deoxyribonucleoside triphosphates coupled to molecules that prevent the leakage of cellular materials from said cells;
- d) contacting said solution of cells containing amplified HIV-1 RNA sequence with labeled nucleic acid probes complementary to said RNA sequences; and
- e) detecting said labeled HIV-1 RNA nucleic acid probes by fluorescence activated flow cytometry so to identify cells containing said HIV-1 RNA sequences.

15. The method of Claim 14 wherein said fixative agent is STF (Streck Laboratories).

16. The method of Claim 14 wherein said deoxyribonucleoside triphosphates is coupled to digoxigenin.

17. The method of Claim 16 wherein said triphosphate compound is dUTP.

18. A method to detect specific preselected amplified nucleic acid sequences in cells comprising:

- a) isolating a class of cells;
- b) treating said cells to prevent diffusion of nucleic acid sequences during amplification out of said cells;
- c) amplifying said specific nucleic acid sequences;
- d) contacting said solution of cells containing amplified nucleic acid with labeled nucleic



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acid probes complementary to said specific nucleic acid sequences;

- e). detecting said labeled nucleic acid probes by fluorescence activated flow cytometry so to identify cells containing said preselected nucleic acid sequences.

## AMENDED CLAIMS

[received by the International Bureau  
on 05 October 1993 (05.10.93):  
original claims 4 and 12 cancelled;  
claims 5-18 renumbered as claims 4-16  
wherein claims 1,4,9,11 and 12 are amended (3 pages)]

1. A method to detect specific preselected nucleic acid sequences in a cell by fluorescence activated cytometry comprising the steps of:
  - a) isolating peripheral blood mononuclear cells;
  - b) incubating said cells with a water soluble fixative to form a suspension;
  - c) amplifying specific nucleic acid sequences in said suspension of cells in the presence of deoxyribonucleoside triphosphates analogs wherein said analogs have substituent groups which prevent diffusion of amplified products containing them from said cells;
  - d) contacting said solution of cells containing amplified nucleic acid with labeled nucleic acid probes complementary to said specific nucleic acid sequences; and
  - e) detecting said labeled nucleic acid probes by fluorescence activated flow cytometry so to identify cells containing said preselected nucleic acid sequences.
2. The method of Claim 1 wherein said class of cells is peripheral mononuclear cells.
3. The method of Claim 1 wherein said water soluble fixative is STF (Streck Laboratories).
4. The method of Claim 1 wherein said deoxyribonucleoside triphosphate is dUTP.
5. The method of Claim 1 wherein said specific nucleic acid is HIV-1 proviral DNA.
6. The method of Claim 1 wherein said nucleic acid is DNA.

7. The method of Claim 1 wherein said nucleic acid is RNA.

8. The method of Claim 1 wherein said specific nucleic acid is HIV-1 RNA.

9. A method to detect HIV-1 proviral DNA sequences in peripheral mononuclear cells by fluorescence activated flow cytometry comprising the steps of:

- a) isolating said peripheral mononuclear cells;
- b) incubating said peripheral mononuclear cells with a water soluble fixative to form a suspension;
- c) amplifying said HIV-1 proviral DNA sequence in said solution of cells in the presence of deoxyribonucleoside triphosphates analogs wherein said analogs have substituent groups which prevent leakage of amplified products containing them from the cell;
- d) contacting said suspension of cells containing amplified HIV-1 proviral DNA sequence with labeled nucleic acid probes complementary to said nucleic acid sequences; and
- e) detecting said labeled HIV-1 proviral nucleic acid probes by fluorescence activated flow cytometry so to identify cells containing said HIV-1 proviral DNA sequences.

10. The method of Claim 9 wherein said fixative agent is STF (Streck Laboratories).

11. The method of Claim 9 wherein said deoxyribonucleoside triphosphate is dUTP.

12. A method to detect HIV-1 proviral RNA sequences in peripheral mononuclear cells by fluorescence activated flow cytometry comprising the

acid probes complementary to said specific nucleic acid sequences;

- e) detecting said labeled nucleic acid probes by fluorescence activated flow cytometry so to identify cells containing said preselected nucleic acid sequences.

## STATEMENT UNDER ARTICLE 19

The amendment of claims 1, 4, 9, 11 and 15 is made in order to more clearly distinguish these claims from International Conference on AIDS, Volume 7, No. 1, issued 16-21 June 1991, N. Cost Taveira et al. "Detection of HIV-1 DNA by PCR Incorporation of Digoxigenin-dUTP and Hybridization to Immobilized Probes", page 115, Abstract No. M.A. 1093, Cytometry, Supplement 4 (Abstracts for the XIV International Meeting of the Society for Analytic Cytology), issued 18-23 March 1990, Timm et al., "Genotyping Cells by Flow Cytometry Using PCR Techniques", page 79, Abstract 481B., and Biological Chemistry Hoppe-Seyler, Volume 371, issued October 1990, R. Seibl et al., "Non-radioactive Labeling and Detection of Nucleic Acids III. Applications of the Digoxigenin System," pages 939-951, especially pages 941 and 948. All of these documents were cited in the international search report as being of particular relevance to the claimed invention. Full support for the amendments to these claims can be found on page 5, lines 7-35, page 6, line 9, page 7, lines 33-34 and page 8, lines 1-3.

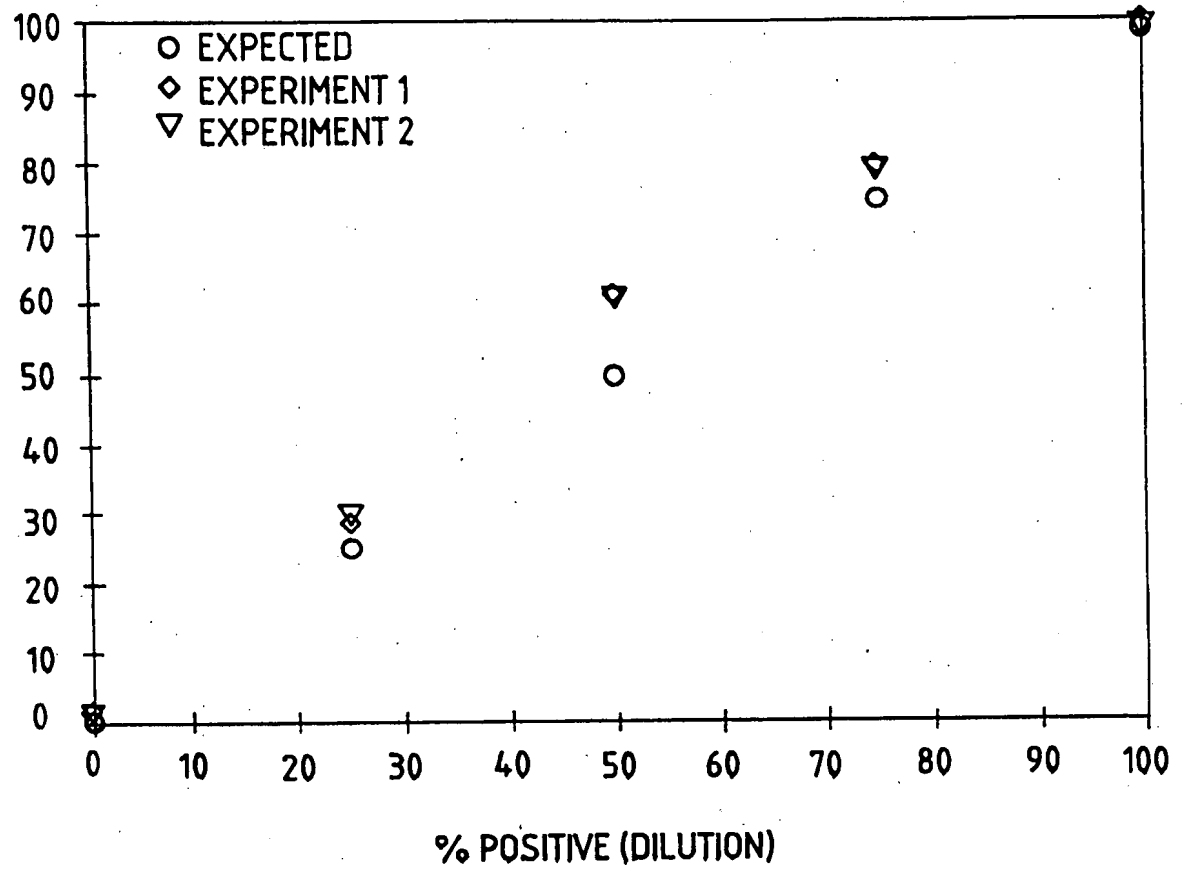
1/3

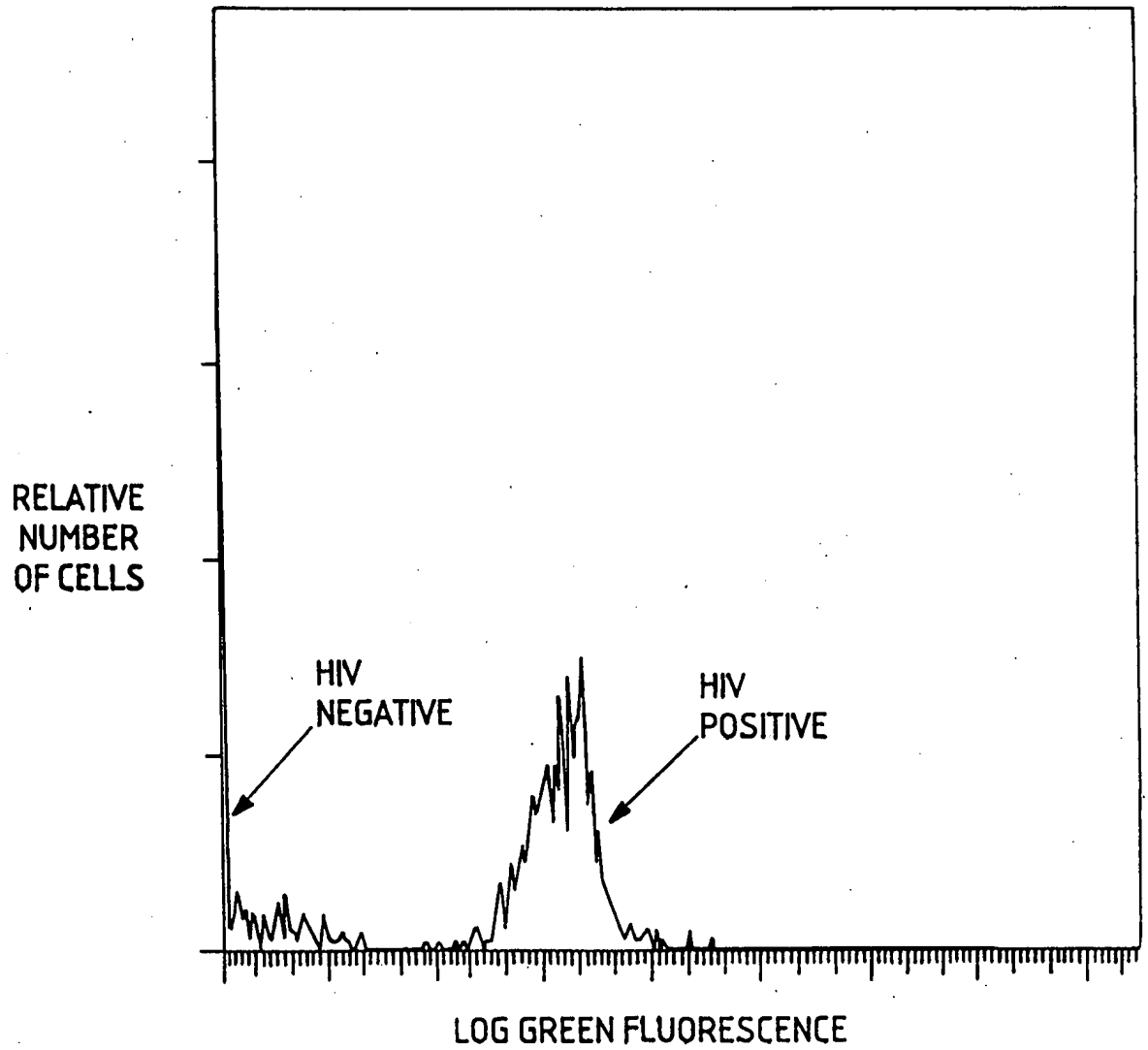
**Fig. 1**



Fig. 2

% POSITIVE  
(MEASURED)



**Fig. 3**



<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC(5) : C12Q 1/68, 1/70; C12P 19/34 US CL : 435/5, 6, 91 According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/5, 6, 91  Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) CA, BIOSIS, MEDLINE, WPI, APS, search terms: fluorescence activated cytometry, amplification, PCR, digoxigenin, STE, HIV-1, DNA, RNA, mononuclear cells, dUTP		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	International Conference on AIDS, Volume 7, No. 1, issued 16-21 June 1991, N. Costa Taveira et al, "Detection of HIV-1 DNA by PCR incorporation of digoxigenin-dUTP and hybridization to immobilized probes.", page 115, Abstract no. M.A.1093.	1-18
Y	Cytometry, Supplement 4 (Abstracts for the XIV International Meeting of the Society for Analytical Cytology), issued 18-23 March 1990, Timm et al, "Genotyping cells by flow cytometry using PCR techniques", page 79, Abstract 481B.	1-18
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
•	Special categories of cited documents:	
*A*	document defining the general state of the art which is not considered to be part of particular relevance	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*E*	earlier document published on or after the international filing date	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*L*	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*O*	document referring to an oral disclosure, use, exhibition or other means	*Z* document member of the same patent family
*P*	document published prior to the international filing date but later than the priority date claimed	
Date of the actual completion of the international search	Date of mailing of the international search report	
22 August, 1993	<b>AUG 30 1993</b>	
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer  MARGARET PARR	
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196	

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biological Chemistry Hoppe-Seyler, Volume 371, issued October 1990, R. Seibl et al, "Non-radioactive Labeling and Detection of Nucleic Acids III. Applications of the Digoxigenin System", pages 939-951, especially pages 941 and 948.	1-18